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| 学 位 論 文 題 目 | Comparative study on chemical constituents of three Gentiana drugs, Gentianae Scabrae Radix, Gentianae Macrophyllae Radix and Gentianae Radix, and their anti-inflammatory activity (3 種類の Gentiana 属生薬「竜胆」、「秦艽」及び「ゲンチアナ」の含有成分とそれらの抗炎症活性に関する比較研究) |
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論文内容の要旨

The genus *Gentiana* (Gentianaceae) consists of approximately 360 species. Many plants from this genus have been widely used in traditional medicine. There are three *Gentiana* drugs recorded in the Chinese Pharmacopoeia (CP), Japanese Pharmacopoeia (JP) and Japanese standards for non-pharmacepoeial crude drugs (Non-JPS), namely *Gentianae Scabrae Radix* (GSR), *Gentianae Macrophyllae Radix* (GMR) and *Gentianae Radix* (GR). GSR is prescribed as the root and rhizome of *G. scabra*, *G. manshurica*, *G. triflora* and *G. rigescens* in CP mainly for the treatment of hepatitis, cholecystitis and allergic inflammations. The former three are also recorded in the JP. GMR is prescribed as the root of *G. macrophylla*, *G. straminea*, *G. crassicaulis* and *G. dahurica* in CP and Non-JPS, mainly used for treatment of various inflammatory diseases such as rheumatoid arthritis. GR is prescribed as the root and rhizome of *G. lutea* in JP and European Pharmacopoeia, mainly used for the treatment of insufficient gastric secretions, intestinal and gastric inflammation, etc. The different uses of each *Gentiana* drug also show up in traditional medicine for the preparation, e.g. GSR being included in heat-clearing prescription “Longdan-Xiegan-tang”, GMR being included in damp-clearing prescription “Duhuo-Jisheng-tang”, and GR being used in the preparation of bitter tonic. GSR sometimes used as a substitution of GR is also recorded.

Previous studies on *Gentiana* drugs from different species revealed a series of iridoid and secoiridoid glycosides, with common principles as gentiopicroside, sweroside and swertiamarin. However, the variation in their chemical compositions, especially the species-specific components of them related to therapeutic effects remains unclear to date. Therefore it is necessary to explore deeply for the sub-major or minor constituents, which might be species-specific to be responsible for bio-activities and/or used as markers for identification. From a phytochemical viewpoint, chemical constituents with anti-inflammatory activity in *Gentiana* drugs are obscure and detailed phytochemical comparison among them is lacking. This study aims to investigate the chemical constituents of each *Gentiana* drug and comparatively analyze their chemical composition, as well as evaluate anti-inflammatory activity *in vitro* for the isolated compounds.

1. Phytochemical investigation and anti-inflammatory activity of GSR, the root and rhizome of *G. scabra*¹⁾

The botanical source of used GSR was identified as *G. scabra* by genetic analysis

of nucleotide sequence of rDNA ITS region. Crude chloroform and methanol extracts of the identified GSR were priorly screened for their inhibitory effect against LPS-induced NO, IL-6 and TNF- α productions *in vitro*. The chloroform extract was found to possess potential anti-inflammatory activity (IC_{50} 112.80 μ g/mL of NO inhibition and 176.81 μ g/mL of IL-6 inhibition, respectively), which was therefore taken for further investigation. The phytochemical investigation on this bioactive extract led to isolation of 19 secoiridoid glycosides (**1-19**), including seven new compounds (**1-5**, **7**, **10**); as well as two lignans (**20**, **21**), three triterpenoids (**22-24**) and four compounds of other types (**25-28**) (Chart 1). Among the known compounds, nine compounds (**6**, **13**, **15**, **17**, **18**, **22-25**) are isolated from this plant for the first time. The secoiridoids as the representative constituents were taken for assay of inhibitory effect on LPS-induced NO, IL-6 and TNF- α productions *in vitro*. 8-*epi*-kingiside derivatives **1-3**; kingiside derivatives **4-6**; and sweroside derivative **10** showed inhibition activity against IL-6 production with IC_{50} of 51.70–63.80 μ M, whereas sweroside derivatives **12** and **15-19** and one swertiamarin derivative **13** showed inhibition effects on both of NO and IL-6 productions with IC_{50} of 60.55–94.95 μ M and 48.91–75.45 μ M, respectively. All the test compounds exhibited weak inhibitory activity (IC_{50} > 100 μ M) in the case of TNF- α bioassay.

2. Phytochemical investigation and anti-inflammatory activity of GMR, the root of *G. crassicaulis* ²⁾

The botanical source of used GMR was identified as *G. crassicaulis* by genetic analysis of nucleotide sequence of rDNA ITS region. Crude chloroform and methanol extracts of the identified GMR, as well as the water-soluble and 30%, 60%, 90% aqueous methanol eluate fractions obtained from the methanol extract through a macroporous resin fractionation procedure were priorly screened for their inhibitory effect against LPS-induced NO and IL-6 productions *in vitro*. Among the extracts and fractions, 30% and 60% aqueous methanol eluate fractions were found to possess the most promising anti-inflammatory activity, which were therefore taken for further investigation. The phytochemical investigation on these two bioactive fractions led to isolation of 20 secoiridoid glycosides (**7-9**, **29-45**), including five new compounds (**29-31**, **41**, **42**); as well as four lignans (**21** and **46-48**), one C-glucoflavonoid (**49**) and seven compounds of other types (**50-56**) (Chart 2). Among the isolated compounds, gentiananosides A (**41**) and B (**42**) were concluded to be novel secoiridoid glycosides with an ether linkage between C-2' of the sugar moiety and C-3 of the aglycone. 18 known compounds (**32**, **34**, **35**, **38-40**, **43-47**, **49-55**) are isolated from this plant for the first time. Compounds **29-33**, **36**, **39** and **40** exhibited inhibitory effects on both NO and IL-6 productions with IC_{50} of 79.88–95.02 μ M and 70.62–77.42 μ M, whereas **37**, **41** and **LA** exhibited inhibitory effects against only IL-6 production with IC_{50} of 75.35, 88.09 and 51.28 μ M, respectively. The remaining compounds exhibited weak inhibitory activity (IC_{50} > 100 μ M) against NO or IL-6 production.

3. Chemical constituents of GR, the root of *G. lutea*, and comparison of chemical composition among GSR, GMR and GR

The botanical source of used GR was morphologically identified as *G. lutea*. The chemical investigation of the methanol extract of GR led to isolation of 20 compounds, including 11 secoiridoids (**9**, **36**, **37**, **43**, **44**, **57-62**), six xanthenes (**63-68**), one C-glucoflavonoid (**69**), one lignan (**21**) and one methyl benzoate derivative (**27**) (Chart 3). All the isolated compounds from the three identified *Gentiana* drugs above were used for comparative analysis to explore their potential chemical marker(s) by HPLC method.

HPLC profiles of GSR, GMR and GR revealed six major common peaks which were identified as loganic acid (**LA**), gentiopicroside (**9**), 6'-O- β -D-glucopyranosyl gentiopicroside (**33**), swertiamarin (**36**), and sweroside (**37**), respectively. The chemical composition of GR obviously differed from those of GSR and GMR in containing xanthenes (**63-68**), which could be probably used as chemical markers. GMR containing macrophyllaside D (**54**), a 2-methoxyanofinic acid derivative belonging to chromenes, clearly differed from GSR containing a group of acetylated and/or benzoylated secoiridoid glycosides (**11-17**) (Chart 4). Besides, some identified common compounds from GSR and GMR also differed in their contents such as **LA**, **9**, **33**, **34** and **37**.

Conclusion

In the current study, 70 compounds, including 12 new ones, have been isolated from the identified GSR, GMR and GR. They are mainly the secoiridoids, as well as xanthenes, C-glucoflavonoids, lignans, triterpenoids and compounds of other types. Secoiridoids as the representative constituents from GSR and GMR exhibited moderate inhibitory effects against LPS-induced NO and IL-6 productions *in vitro*, however they are attractive in their structural diversity. The HPLC profiles of the three *Gentiana* drugs showed high similarities, whereas phytochemical characteristics of each drug was observed, such as the acetylated and/or benzoylated secoiridoids in GSR, 2-methoxyanofinic acid derivatives in GMR, and xanones in GR, which could be used as candidate markers for authentication and standardization of *Gentiana* drugs. Additionally, the chemical diversity elucidated by this study suggests that these three *Gentiana* drugs might have their own pharmacological effects, which imply that the substitution among them should be paid attention.

Reference

- 1) He YM, Zhu S, Ge YW, Kazuma K, Zou K, Cai SQ, Komatsu K. The anti-inflammatory secoiridoid glycosides from *Gentianae Scabrae Radix*: the root and rhizome of *Gentiana scabra*. J Nat Med, DOI 10.1007/s11418-015-0894-8
- 2) He YM, Zhu S, Ge YW, Cai SQ, Komatsu K. Secoiridoid glycosides from the root of *Gentiana crassicaulis* with inhibitory effects against LPS-induced NO and IL-6 productions in RAW264 macrophages. J Nat Med, (accepted).

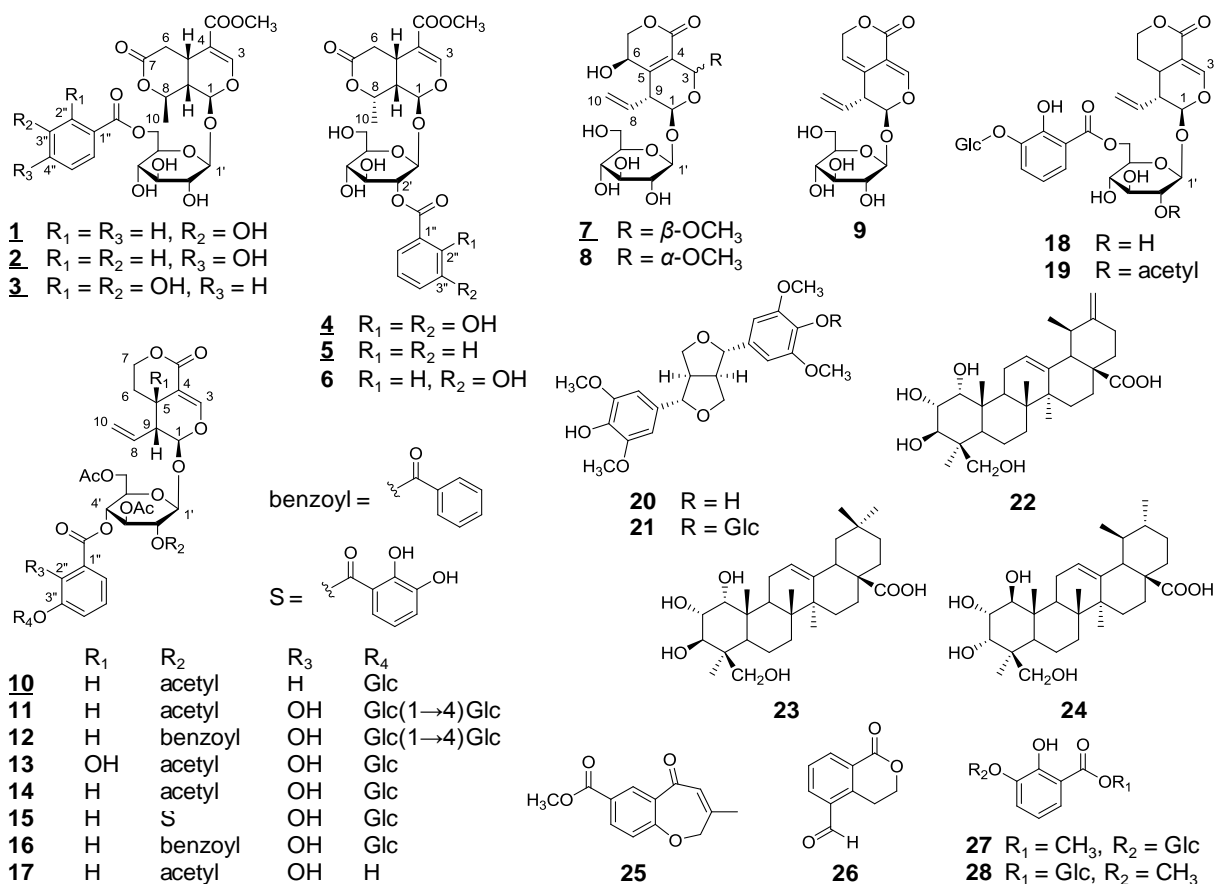


Chart 1 Structures of compounds isolated from GSR, the root and rhizome of *G. scabra* (new compound)

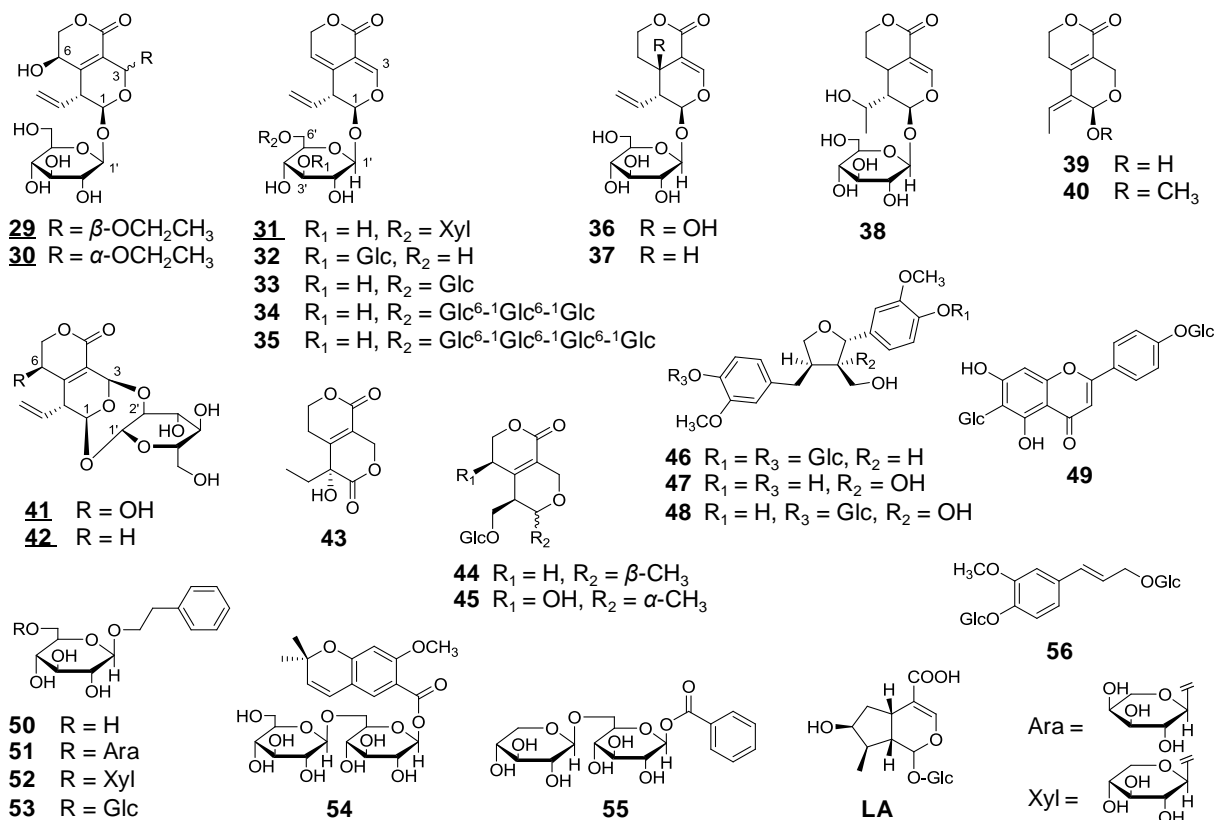


Chart 2 Structures of compounds isolated from GMR, the root of *G. crassicaulis* (new compound)

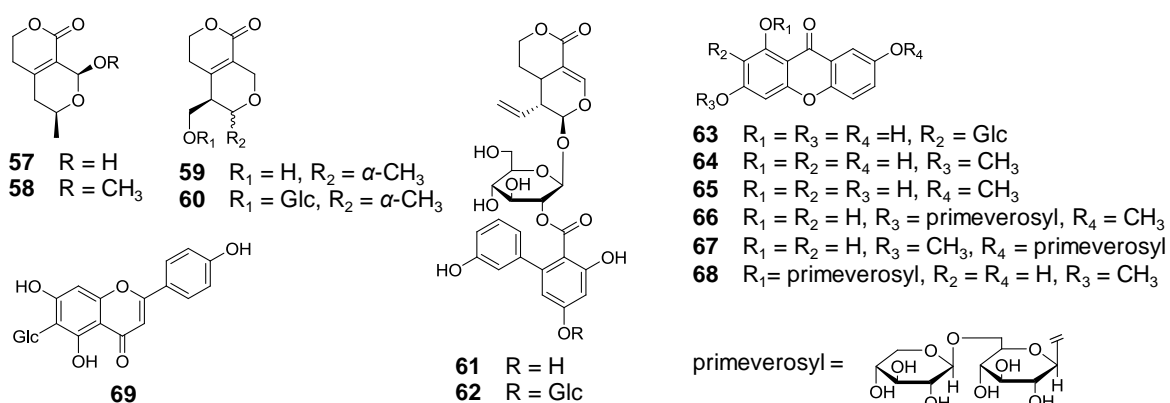


Chart 3 Structures of compounds isolated from GR, the root and rhizome of *G. lutea*

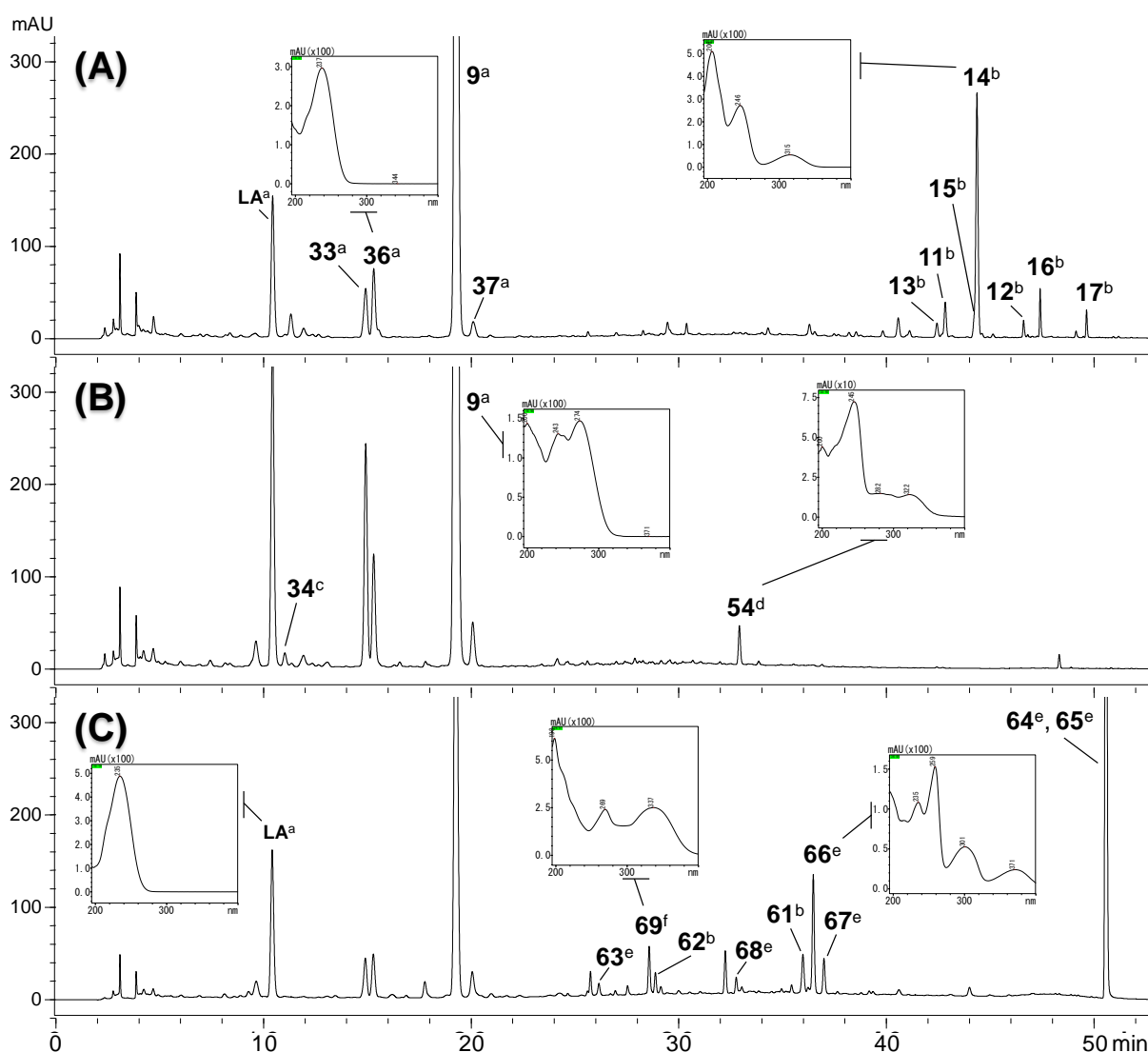


Chart 4 Representative HPLC chromatograms of identified GSR, GMR and GR (254 nm)

(A): GSR, the root and rhizome of *G. scabra*; **(B):** GMR, the root of *G. crassicaulis*; **(C):** GR, the root and rhizome of *G. lutea*. Six classes of characteristic compounds : a, the common principal secoiridoids glycosides; b, acetylated/benzoylated secoiridoid glycosides; c, polyglycosylated secoiridoid glycosides; d, 2-methoxyanofinic acid derivative; e, xanthones, characteristic constituents for GR; f, C-glycoflavonoids.

学 位 論 文 審 査 の 要 旨

日本で使用されるリンドウ科の*Gentiana*属生薬には、漢方方剤に配合される竜胆と秦艽家庭薬原料とされるヨーロッパ生薬のゲンチアナがあり、『日本薬局方』または『日本薬局方外生薬規格2012』では、竜胆は*Gentiana scabra*、*G. manshurica*及び*G. triflora*の根及び根茎、秦艽は*G. macrophylla*、*G. straminea*、*G. crassicaulis*及び*G. dahurica*の根ゲンチアナは*G. lutea*の根茎及び根が規定される。中国医学では、竜胆は清熱薬として肝炎、慢性胃炎、尿道炎などに、秦艽は去風湿薬として関節リウマチ、四肢の麻痺などに応用される。一方、ゲンチアナは収斂薬、健胃薬などとされ、3種類の生薬の用途には類似性と相違性が混在して見られる。これまでの研究により、3生薬の主成分はセコイリドイド配糖体のgentiopicrosideであることが知られているが、各生薬に固有で、薬効の違いに関与する成分は明らかになっていない。また、竜胆と秦艽では抗炎症作用が重要であるが、活性を有する化合物は不明瞭である。

そこで、申請者は、*Gentiana*属3生薬に含有される化学成分を明らかにし、単離した化合物の抗炎症作用を評価すること、及び各生薬に特徴的な成分を明らかにすることを目的として本研究を行い、下記に示す知見を得た。

1. 竜胆 (*G. scabra*) に含有される化合物とそれらの抗炎症作用

竜胆のクロロホルムエキスは、マウスマクロファージ様細胞株RAW264細胞系で、LPS誘導によるサイトカインIL-6の産生及び一酸化窒素(NO)の産生の抑制作用を示したため、このエキスについてカラムクロマトグラフィー及び分取HPLCを行って分画・分離し、新規7化合物を含むセコイリドイド配糖体19化合物、リグナン2化合物、トリテルペノイド3化合物及びその他4化合物を単離し、構造決定した。セコイリドイド配糖体について、リウマチ性関節炎と関連性のあるIL-6、TNF- α の産生抑制作用及びNO産生抑制作用を調べた結果、8-*epi*-kingisideの類縁体3化合物とkingisideの類縁体3化合物、及びswerosideの類縁体1化合物が中程度のIL-6産生抑制作用(IC₅₀ 51.7- 63.8 μ M)を示した。また、swerosideの類縁体6化合物とswertiamarinの類縁体1化合物は中程度のIL-6産生抑制作用とNO産生抑制作用を示した。TNF- α 産生抑制作用はすべての化合物で弱かった。IL-6及びNO産生抑制作用には、セコイリドイド配糖体のアグリコンのC-5位の水酸基、糖部のC-2'位のベンゾイル基の存在が重要である可能性が示唆された。

2. 秦艽 (*G. crassicaulis*) に含有される化合物とそれらの抗炎症作用

秦艽をクロロホルムで抽出後、残渣にメタノールを加えて抽出したエキスの30%及び60%メタノール溶出画分においてIL-6産生及びNO産生の抑制作用が認められたことから、これらの画分をさらに各種溶媒で分画して、新規5化合物を含むセコイリドイド20化合物、リ

グナン4化合物、フラボノイドC配糖体1化合物、loganic acid、その他7化合物を単離し、構造決定した。新規化合物のgentiananaside A、gentiananaside Bは糖部のC-2'位とアグリコンのC-3位がエーテル結合した新型のセコイリドイド配糖体であった。セコイリドイドのうち、gentiananaside C、gentiananaside D、6'-O-β-D-xylopyranosylgentiopicoside、olivieroside C、6'-O-β-D-glucopyranosylgentiopicoside、swertiamarin、8-hydroxy-10-hydrosweroside、swerimilegenin H、swerimilegenin Iは中程度のIL-6産生抑制作用とNO産生抑制作用を示し、一方、swerosideとgentiananaside A、及びloganic acid (IC₅₀ 51.3 μM) はIL-6産生抑制作用のみを示した。

3. ゲンチアナ (*G. lutea*) に含有される化合物と、3 生薬 (竜胆、秦艽、ゲンチアナ) の成分比較

ゲンチアナのメタノールエキスから、セコイリドイド 11 化合物、キサントン 6 化合物及びフラボノイド C配糖体、リグナン、メチルベンゾエート類縁体各 1 化合物を単離、同定した。3 生薬は共通して gentiopicoside、6'-O-β-D-glucopyranosylgentiopicoside、swertiamarin、sweroside 及び loganic acid を含有したが、秦艽は gentiopicoside 及び 6'-O-β-D-glucopyranosyl -gentiopicoside 及び loganic acid の含量が高かった。竜胆は糖部がアセチル化またはベンゾイル化したセコイリドイド配糖体、秦艽はクロメン、ゲンチアナはキサントンを含有することにより明らかに区別された。

以上、3生薬から新規12化合物を含む70化合物を単離、同定した。セコイリドイド42化合物のうち25化合物は中程度のIL-6産生抑制作用を示し、そのうち16化合物はNO産生抑制作用も示すことが明らかになり、竜胆と秦艽の抗炎症作用が裏付けられた。一方、3生薬の特徴的な成分組成が明らかになり、これらを指標にすることによりそれぞれの生薬の標準化が行えること、及び代替資源植物を開発できることが示唆できた。これらの研究成果は、学位論文として十分に評価し得るものである。

主査及び副査は、申請者 何 毓敏 の論文内容について審査を行うとともに面接試験を行い、博士 (薬学) を授けるに値するものと判定した。

1. He Y.M., Zhu S., Ge Y.W., Kazuma K., Zou K., Cai S.Q., Komatsu K.: The anti-inflammatory secoiridoid glycosides from *Gentianae Scabrae Radix*: the root and rhizome of *Gentiana scabra*. J. Nat. Med., 2015. DOI 10.1007/s11418-015-0894-8
2. He Y.M., Zhu S., Ge Y.W., Cai S.Q., Komatsu K.: Secoiridoid glycosides from the root of *Gentiana crassicaulis* with inhibitory effects against LPS-induced NO and IL-6 production in RAW264 macrophages. J. Nat. Med., 2015. DOI 10.1007/s11418-015-0903-y